REFLECTIVE PROPERTIES OF IRIDOPHORES AND FLUORESCENT 'EYESPOTS' IN THE LOLIGINID SQUID *ALLOTEUTHIS SUBULATA* AND *LOLIGO VULGARIS*

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Summary

Observations were made of the reflective properties of the iridophore stripes of the squid *Alloteuthis subulata* and *Loligo vulgaris*, and the likely functions of these stripes are considered in terms of concealment and signalling.

In both species, the mantle muscle is almost transparent. Stripes of iridophores run along the length of each side of the mantle, some of which, when viewed at normal incidence in white light, reflect red, others green or blue. When viewed obliquely, the wavebands best reflected move towards the blue/ultraviolet end of the spectrum and their reflections are almost 100 % polarised. These are properties of quarter-wavelength stacks of chitin and cytoplasm, predicted in theoretical analyses made by Sir A. F. Huxley and Professor M. F. Land. The reflecting surfaces of the individual iridophores are almost flat and, in a given stripe, these surfaces are within a few degrees of being parallel. Both species of squid have conspicuous, brightly coloured reflectors above their eyes. These 'eyespots' have iridescent layers similar to those found on the mantle but are overlaid by a green fluorescent layer that does not change colour or become polarised as it is viewed more obliquely. In the sea, all reflections from the iridophore stripes will be largely confined to the blue-green

parts of the spectrum and all reflections in other wavebands, such as those in the red and near ultraviolet, will be weak. The functions of the iridophores reflecting red at normal incidence must be sought in their reflections of blue-green at oblique angles of incidence. These squid rely for their camouflage mainly on their transparency, and the ventral iridophores and the red, green and blue reflective stripes must be used mainly for signalling. The reflectivities of some of these stripes are relatively low, allowing a large fraction of the incident light to be transmitted into the mantle cavity. Despite their low reflectivities, the stripes are very conspicuous when viewed from some limited directions because they reflect light from directions for which the radiances are much higher than those of the backgrounds against which they are viewed. The reflective patterns seen, for example, by neighbouring squid when schooling depend on the orientation of the squid in the external light field and the position of the squid relative to these neighbours.

Key words: squid, *Alloteuthis subulata*, *Loligo vulgaris*, iridophore, reflectivity, signalling, concealment, fluorescence, quarter-wavelength stack.

Introduction

The two species of squid on which these observations were made live at a wide range of depths. *Alloteuthis subulata* is found between close to the sea surface and 100 m depth, whereas *Loligo vulgaris* is caught as deep as 400 m (M. R. Clarke, personal communication). These squid are schooling animals that need to signal changes in relative position to neighbouring squid to maintain the coherence of the school. They are, however, also heavily predated and might therefore be expected to have efficient camouflage systems. Squid are renowned for their ability to express a wide variety of chromatic, postural and locomotor components for camouflage and signalling (Hanlon, 1982; Hanlon, 1988; Cornwell et al., 1997; Hanlon et al., 1999). The neurally controlled chromatophore organs in the skin of cephalopods contribute substantially to producing a large number of body patterns,

which are used for very effective concealment as well as for visual signalling. The importance of chromatophores for camouflage and communication has been documented extensively (for a review, see Hanlon and Messenger, 1996).

Internal to the layer of chromatophore organs in the skin squid possess light-reflecting cells (iridophores) (Williams, 1909). There has been very little work on these cells. Most of the studies have focused on morphological (Arnold, 1967; Mirow, 1972; Arnold et al., 1974) and physiological (Cooper and Hanlon, 1986; Cooper et al., 1990; Hanlon et al., 1990) aspects of squid iridophores. Here, we show that the squid *A. subulata* and *L. vulgaris* possess distinct stripes of iridophores that reflect different wavebands when viewed in white light. Some squid iridophores have plates which have been shown by interference microscopy to have refractive indices (*n*) close
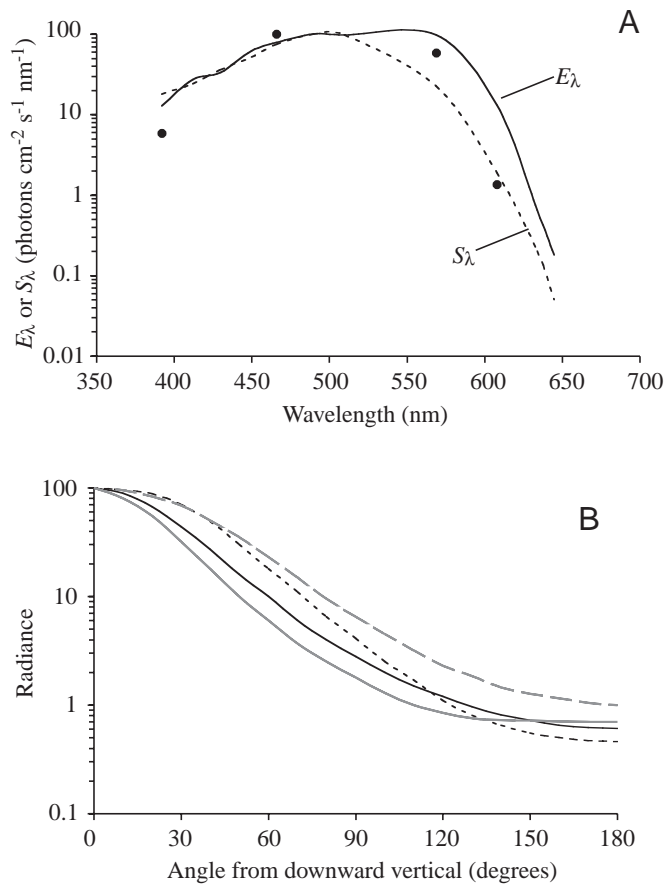
Fig. 1. (A) Spectral irradiance ($E\lambda$) of downwelling daylight for the Gulf of California at 19 m depth (solid line) (after Tyler and Smith, 1970) and for the English Channel at 20 m depth (filled circles) (after Atkins, 1945). Spectral absorption ($S\lambda$) of the squid rhodopsin (broken line) for a maximum optical density of 0.5 (calculated after Knowles and Dartnall, 1977) with the wavelength for maximum absorption (λ_{max}) at 499 nm (Morris et al., 1993). Maximum values have been made equal to 100. (B) Angular distribution of daylight in the sea: grey solid line, after an equation and constants suggested to one of us by Dr J. E. Tyler for oceanic waters off the Azores (see Denton et al., 1972); short dashed line, for Lake Pend Oreille (Tyler, 1960); long dashed line, for turbid water at a depth of 40 m off Japan (Sasaki et al., 1962, cited in Jerlov, 1976); black solid line, for Mediterranean waters (Lundgren, 1976, cited in Jerlov, 1976). Radiances for 0° (vertically downwards) have been made equal to 100.

to 1.56, which is the refractive index of chitin, whilst the optical thicknesses were those expected for quarter-wavelength ($\lambda/4$) stacks of alternating chitin and cytoplasm plates (Denton and Land, 1971). As shown by Huxley (Huxley, 1968) and Land (Land, 1972), light reflected from an ideal $\lambda/4$ multilayer stack, for which the optical thicknesses of the plates and spaces are a quarter of the wavelength best reflected at normal incidence, can be characterised as follows. (i) Effect of oblique incidence: as the angle of the light incident to a multilayer reflector is made more oblique, the waveband of the reflected light moves towards the short (blue/ultraviolet) end of the spectrum; (2) Polarisation: depending on the angle of

incidence, the light reflected from a $\lambda/4$ stack will be plane polarised to a greater or lesser extent. At small angles of incidence, the reflected light is hardly polarised whilst at Brewster's angle (49.5° for chitin and water) the reflected light is completely polarised. Stacks that are not ideal have very similar reflective properties as long as each pair of plates and spaces has an optical thickness of $\lambda/2$ and the thickness of a plate is not less than $\lambda/8$ (Denton and Land, 1971).

To reduce visibility and send out visual signals in particular directions, squid exploit the special properties of light in the sea. These properties differ in a number of aspects from those found on land.

The intensity of terrestrial daylight changes little over the waveband 400–700 nm (i.e. near-ultraviolet to deep red) (Le Grand, 1952). Sea water, however, absorbs and scatters light selectively and, with increasing depth, the wavebands of light transmitted are increasingly confined to wavelengths between 400 and 580 nm (Atkins, 1945; Tyler and Smith, 1970; Jerlov, 1976). Measurements of the spectral irradiance of waters close to Plymouth were made by Atkins (Atkins, 1945). More detailed measurements on very similar water masses are given by Tyler and Smith (Tyler and Smith, 1970). Both sets of measurements are shown in Fig. 1A. It can be seen that even at depths as shallow as 19 m the wavebands are largely confined to the blue-green parts of the spectrum, the cut-off being much sharper on the red side of the spectrum than on the blue side. Like many marine animals, the three species of squid used in our experiments have visual pigments that absorb best in the blue-green. For *A. subulata*, maximal absorption (λ_{max}) is at 499 nm, and for *Loligo forbesi* λ_{max}=494 nm (Morris et al., 1993) (see Fig. 1A).

On a sunny day, the direction of maximal radiance near the sea surface is approximately that to which sunlight is refracted on penetrating into the sea. As we go to greater depths, the angular distribution of daylight approximates to an asymptotic condition, for which the radiance is maximal in a directly downward direction and falls with increase in angle to a minimal value for light travelling directly upwards (Fig. 1B). The radiance found looking directly upwards can be over 100 times greater than the radiance found looking directly downwards (Tyler, 1960; Tyler, 1963; Jerlov and Fukuda, 1960; Jerlov, 1976). Whilst the intensity of light decreases rapidly with increasing depth, its angular distribution is relatively constant. The angular distribution of light depends upon the balance between absorption and scattering of light. To a large degree these vary together so that the distributions change little between the various water masses in which the loliginid squid spend most of their lives. Near the sea bottom, the angular distribution of upwelling light changes in ways that depend on the albedo of the sea bottom (Jerlov, 1976).

Being completely transparent is evidently the ideal solution for camouflage, and very many midwater animals are highly transparent (McFall-Ngai, 1990; Johnson and Widder, 1998). There are, however, some parts of the body, such as the eyes and the inksac, that could not function if they were transparent, so other camouflage methods have evolved in

addition to transparency. When swimming very close to a dark sea bottom, chromatophores most probably play an important role in matching the background. In the midwaters of the sea, where the light intensity changes dramatically depending on the angle of view, pigments such as those contained by chromatophores will make chromatophores very conspicuous when, for example, the squid is seen from below. Light reflectors may be very efficient for camouflage in midwaters and it is important to recall that they may also enable the possessor to give strong visual signals that may be useful, for example, in schooling.

The squid used in our experiments all appeared very transparent, with the chromatophores mostly in a retracted state. When we observed squid in tanks illuminated by white light, the reflectors could easily be seen. The iridophores appeared in stripes above the eyes and on the mantle, and the colours they reflected covered the full extent of our visible spectrum, i.e. from red to deep blue. When the squid were viewed in far blue/ultraviolet light, the stripes above the eyes were seen to fluoresce brightly in the green parts of the spectrum and the emitted green colour was found to be independent of the angle of view. Here, we only describe the reflective patterns produced by the reflective stripes. The complex interactions between chromatophores and iridophores are still under investigation.

Materials and methods

Squid

The squid *Alloteuthis subulata* (Lamarck) (mantle length 10–15 cm) and *Loligo vulgaris* Lamarck (mantle length 20–30 cm) were caught off the Plymouth coast by jigging and trawling and kept in the laboratory's closed circulating seawater system at approximately 12–16 °C. For measurements of spectral reflectivity of the various reflecting structures squid were killed by decapitation. We did not observe an appreciable change in the reflective properties of the iridophore stripes on decapitation of the squid.

Spectral reflectivity, iridophore orientation, transparency and fluorescence

Spectral reflectivities of squid iridophores were measured using the apparatus shown in Fig. 2A. The reflecting material was placed on a small tilting table (Denton and Nicol, 1965). Illumination was by a narrow beam of white light, which was set to give known angles of incidence to the reflecting structures. Perspex wedges could be attached to the side of the tilting table to obtain an angle of 55 ° incidence. Using a dissecting microscope, images of the reflections of a group of iridophores were formed on a small aperture leading to a photomultiplier tube (PMT) (Thorn EMI 9924B). A diaphragm was placed on the objective lens of the dissecting microscope, so that only light over a range of angles of 5 ° could enter the microscope. Interference filters (Balzer), which transmitted only narrow wavebands of light, were placed in the light beam leading to the PMT. Polaroid filters
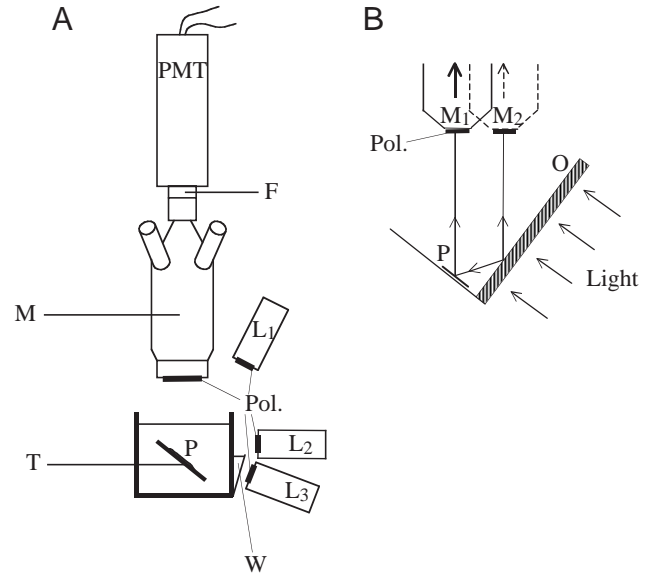

Fig. 2. (A) Apparatus used to measure spectral reflectivity and polarisation. F, interference filters; L_1, L_2, L_3, light sources; M, dissecting microscope; P, preparation; PMT, photomultiplier tube; Pol., polarising filters; T, tilting table; W, Perspex wedge. (B) Apparatus used to measure reflectivity (after Denton and Nicol, 1962). Symbols as in A. O, opal glass; M_1, M_2, dissecting microscopes.

attached to the light sources could be rotated by 90 ° so as to polarise the incident light with its electric vector in either the perpendicular or parallel planes of incidence. A polaroid attached to the base of the dissecting microscope was positioned in the same plane of polarisation as the polaroid of the light source and so acted as an analyser to the reflections from the specimens. Relative reflectivities of the preparations were found by comparing, for each filter, the output of the PMT to light reflected from a specimen with that of a freshly cleaned block of magnesium carbonate, a surface that is known to reflect nearly 100 % of light of all visible wavelengths (Benford, 1947).

We cannot describe the reflective properties of the iridophore stripes in simple terms, because they combine the properties of plane mirrors and scattering surfaces. To give an estimate of reflectivity, some measurements were made on the 'red' and 'green' stripes and the 'eyespots' using the apparatus described by Denton and Nicol (Denton and Nicol, 1962) (Fig. 2B). In this method, the preparation was placed on one side at 90 ° to an opal glass, which scattered light according to the cosine law (Walsh, 1958). The opal glass was illuminated uniformly from the opposite side of the preparation so that all iridophores reflected light into the measuring apparatus, the variations in orientation of the iridophores being matched by the light received from the opal glass at appropriate angles. Reflectivity is given as the ratio of the light entering the PMT from the reflecting material and the opal glass. This measure of reflectivity resembles to a large degree the situation found in nature, in which the iridophores reflect light from a broad source of light. Since the measurements on the tilting table

(Fig. 2A) were made using narrower beams of light than those used on the opal glass chamber (Fig. 2B), changes in reflectivity with changes in angle of incidence were better resolved. However, the measurements made on the opal glass chamber gave better values for reflectivity than those of the tilting table. The curves shown in Fig. 8, Fig. 9 and Fig. 11 are those derived from the tilting table scaled to the values of reflectivity obtained on the opal glass chamber.

To find the orientations of the reflecting structures with respect to the skin surface of a squid, the inside of a squid mantle was lined with black plastic foil to maintain the shape of the mantle. The squid was placed on a tilting table (see Fig. 2A), and the angular settings for the brightest reflections at near normal incidence of the iridophores of the right and left sides of the squid were found. These angular settings were then subtracted from those for the brightest reflections at the same angle of incidence found for a piece of aluminium foil wrapped around the squid adjacent to the area under investigation (Denton and Rowe, 1994).

Transparency was measured with a similar arrangement to that shown in Fig. 2A. The light source was placed below the Perspex box containing the tilting table, so that the light beam passed through the specimen on the tilting table into the PMT. The ratio of light entering the microscope with and without the specimen in the light beam was taken as the measure of transparency. The animals used had not fed prior to the measurements.

To measure fluorescence from the fluorescent layers of the 'eyespots', the fluorescent tissue was homogenised and extracted in ethanol. Excitation and emission spectra for these preparations were determined using a Perkin-Elmer 3000 fluorescence spectrometer.

Photographs of iridophores were taken with a Nikon (UFX-II) and a Pentax camera using 400 and 1600 ASA slide and print films (Fujicolor).

Theoretical calculations of spectral reflectivity

The theoretical calculations of spectral reflectivity were made by D. M. Rowe after equations given by Huxley (Huxley, 1968) and Land (Land, 1972). They include spectral reflectivity for both planes of polarisation of an ideal $\lambda/4$ stack consisting of 10 alternating high ($n=1.56$) and low ($n=1.33$) refractive index platelets.

Results

Theoretical calculations for spectral reflectivity

We give in Fig. 3, as an example of the properties of ideal $\lambda/4$ stacks, the results of theoretical calculations of reflectivity and polarisation for obliquities to 80° incidence of a stack with 10 plates of chitin and spaces of cytoplasm, where $\lambda_{max}=660$ nm. Fig. 3A shows the spectral reflectivity for the incident light polarised in the plane perpendicular to the plane of incidence. It can be seen that the reflected wavebands move towards the blue/ultraviolet end of the spectrum with increasing obliquity of the incident light. In Fig. 3B, the incident light is
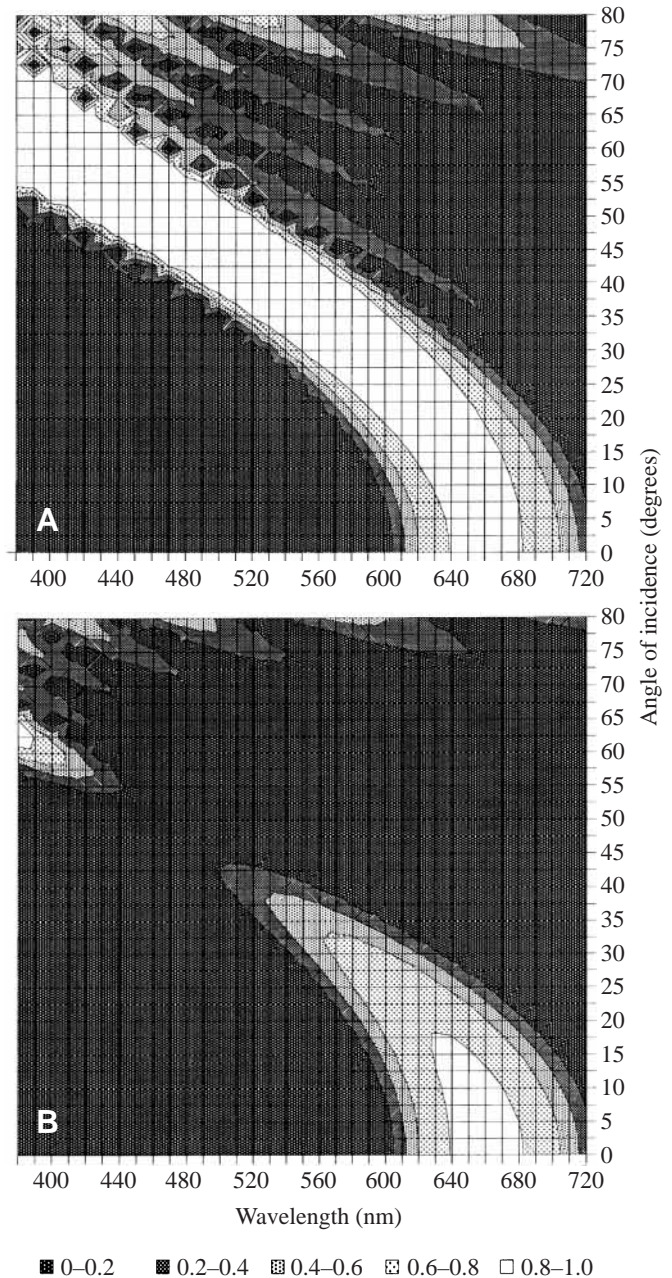

Fig. 3. Calculated spectral reflectivities for an ideal quarter-wavelength ($\lambda/4$) stack with 10 platelets of chitin and spaces of cytoplasm at angles of incidence from 0 to 80° ($\lambda_{max}=660$ nm at normal incidence) (calculated by D. M. Rowe). The degree of shading at each point in the two diagrams represents reflectivity: the lighter the shading, the higher the reflectivity (ranges given below figure). (A) For light polarised in the plane perpendicular to the plane of incidence. (B) For light polarised in the plane parallel to the plane of incidence. Changing the angle of the incident light from 0 to 25° only shifts the wavelengths best reflected from 660 nm to approximately 610 nm, while a change in the incident light from 45 to 55° shifts the wavebands best reflected from 500 to 440 nm.

polarised in the plane parallel to the plane of incidence. At Brewster's angle (49.5°), all reflected light will be polarised in the plane perpendicular to the plane of incidence.
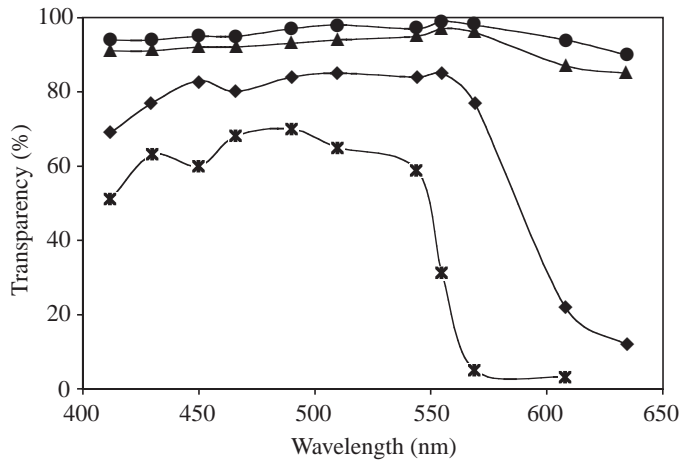
Fig. 4. Transparency in the squid *Alloteuthis subulata*; (✗) measured through the entire mantle in a position anterior to the inksac; (◆) measured through the entire mantle in a position posterior to the inksac; (▲) measured through the mantle wall with intact skin; and (●) measured through the mantle wall from which the skin had been removed.

Transparency

Apart from the eyes and the inksac, the body of a squid is very transparent. In Fig. 4, we show several measurements of transparency of the mantle of *A. subulata*. We found that, if chromatophores were retracted, the entire mantle, with internal organs and skin intact, transmitted on average 57 % (anterior to the inksac) and 80 % (posterior to the inksac) of light in the blue-green parts of the spectrum. The mantle wall with intact skin transmitted 93 % of light in the blue-green, whilst the mantle wall with skin removed transmitted 97 % of blue-green light. The combined losses in blue-green light by absorption, reflection and scattering by the skin and the mantle wall were only 7 % of the incident light. The above measurements show that in the region anterior to the inksac 29 % of the incident blue-green light is lost in the internal organs, while in the region posterior to the inksac only 6 % of the incident blue-green light is lost. Losses were high for light in the red parts of the spectrum.

Spectral reflectivity, polarisation and orientation of the reflecting stripes in squid

The reflective stripes on the mantle and above the eyes of the squid *A. subulata* and *L. vulgaris* were found to be closely similar in position and orientation. These stripes differed in the waveband, intensity, direction and polarisation of the light they reflected. The iridophores making up individual stripes were very similar to each other in size, whilst iridophores differed in size between the stripes. The whole surface of an iridophore within an individual iridophore stripe was almost flat, and in any given area the iridophore surfaces varied by only a few degrees. Fig. 5A shows a diagram of the various reflecting stripes found in the two species. The stripes are named according to the colour they reflected close to normal incidence when viewed in white light (see Fig. 5B for orientation of
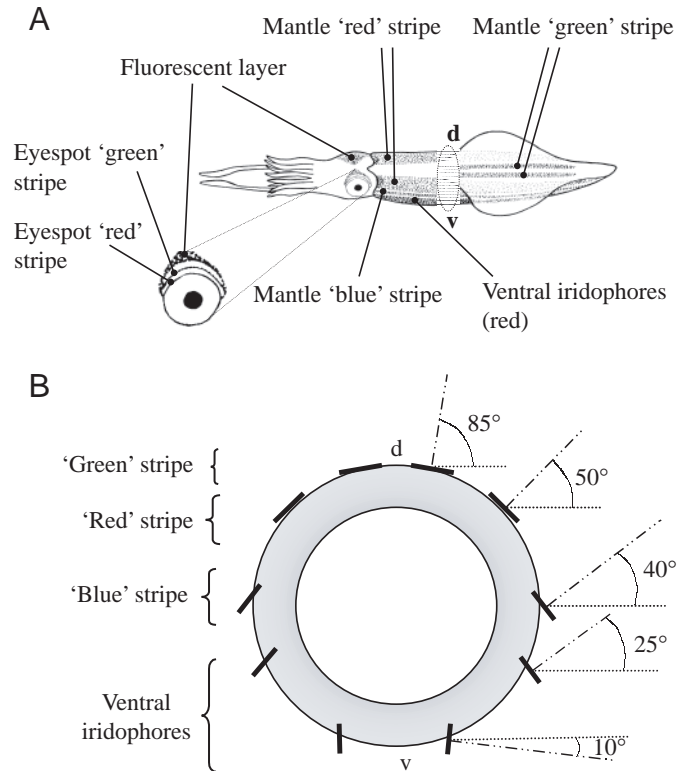

Fig. 5. (A) Reflecting stripes of the squid *Alloteuthis subulata* and *Loligo vulgaris*. The names are given according to the colour reflected when the stripes are viewed in white light at near-normal incidence (see text for details). (B) Cross section perpendicular to the long axis of a squid's mantle, in the region of the fins (dotted oval in A). d and v represent dorsal and ventral regions, respectively. For each stripe, solid lines indicate the iridophores. Broken lines, normal incidence to the iridophores; dotted lines, horizontal.

normal incidence). Fig. 5B shows, in cross section, the mantle of a squid near the fin. It shows the orientations of the iridophores making up the individual iridophore stripes shown in Fig. 5A. The angles indicated are those obtained from the measurements using aluminium foil. The measurements show that the iridophores of the 'green' and 'red' stripes lie almost parallel to the skin surface within which they lie (but see Fig. 6), while those of the 'blue' stripes and the iridophores of the ventral side lie at an angle to the skin surface. The angles indicated are those between the normals of the iridophores and the horizontal.

The mantle
The 'red' stripes

Both species of squid have two 'red' stripes, one on each side, running from the anterior to the posterior end of the mantle. From Fig. 5B, it can be seen that the iridophores of the 'red' stripe lie approximately parallel to the surface of the skin and make an angle of approximately 50 ° with the horizontal. The reflecting structures of the 'red' stripes are lozenge-shaped groups of iridophores, each iridophore being between 200 and 300 µm long. The iridophores are arranged in groups of four.
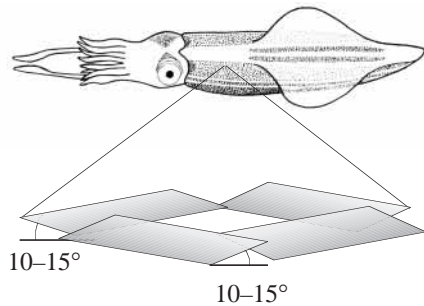
10–15°

10–15°

Fig. 6. Diagram showing the orientation of iridophores of the 'red' stripe on the left side of the mantle. The group is made up of two pairs, each tilted by 10–15° along the antero-posterior plane of the mantle, one pair tilted towards the head, the other towards the 'tail'.

Of each group, the iridophores that are parallel to each other are tilted by 10–15° along the antero-posterior plane of the mantle, one pair being tilted towards the head, the other towards the 'tail'. Fig. 6 shows the arrangement of individual iridophores of such a group for the 'red' stripe on the left side of the mantle. This arrangement is symmetrical with respect to the midline of the squid's mantle. These iridophore groups are very similar in appearance to those shown by Cooper et al. (Cooper et al., 1990) in their figs 1 and 2. The iridophores of this stripe are not in a reflective state at all times: they vary from being overall non-reflective to weakly or strongly reflective and may often be observed in small patches. When in a weakly or strongly reflective state, these iridophores appear red in white light for reflections at around normal incidence. When they are viewed at increasingly oblique angles of incidence, the reflected wavebands change from red to orange to yellow to green and finally to blue at an angle of approximately 55°. At near-normal incidence, reflectivities in the perpendicular and parallel planes of polarisation are almost equal. At high angles of incidence, reflectivities in the perpendicular plane of polarisation are high whilst those in the parallel plane of polarisation are low. Fig. 7A,B,C shows photographs of the 'red' stripe at angles of incidence of 15°, 45° and 55°, respectively. The corresponding curves of spectral reflectivity measured on *L. vulgaris* and *A. subulata* are shown in Fig. 8, with the tilting table set so that the preparation gave the best reflections for one of the two sets of iridophores.

A large fraction of the incident light is transmitted through the spaces between the iridophores and a further fraction through the iridophores. At normal incidence, at which red light is reflected by the iridophores, the transmitted wavebands are in the blue-green. At oblique incidence, the transmitted wavebands are of the longer (red and orange) wavelengths.

Except at the most anterior end, where they are very closely packed, the 'red' stripe iridophores are loosely spaced and their combined area covers approximately half the surface of the stripe. The reflectivities at the anterior end of the mantle are approximately 2–3 times higher than those found at a position halfway along the length of the mantle. When facing the animals from the front, the reflections from the collar

iridophores are very conspicuous. The collar iridophores and the remainder of the 'red' stripe are not always in a reflective state at the same time: they have been observed with the 'red' stripe being non-reflective.

The 'green' stripes

There are two very bright reflecting stripes between the fins on the dorsal side of the mantle of *L. vulgaris* (Fig. 5). These have been described as 'dorsal iridophore sheen' (e.g. Hanlon, 1982; Hanlon et al. 1999). They are not present in *A. subulata*. These stripes are densely packed with iridophores, which are 100–200 µm long. The iridophores of this stripe have been observed to extend some way towards the anterior end of the mantle, and small 'patches' of green iridescence can sometimes be observed above the 'red' stripes on the dorsal side. These patches, described as 'iridophore splotches' (e.g. Hanlon, 1982), have spectral reflectivity and polarisation characteristics identical to those of the 'green' stripes. In Fig. 5B, it can be seen that the iridophores of this stripe lie approximately parallel to the skin surface, so that their normals make angles of approximately 85° with the horizontal. In white light and at low angles of incidence, these stripes reflect green. At angles between 45 and 55° incidence, the reflections become blue and polarised. Photographs of the reflections are shown in Fig. 7D,E. Fig. 9 shows their spectral reflectivity at three angles of incidence.

The ventral iridophores

The iridophores making up the ventral side are densely packed, approximately 200–300 µm long and 50 µm wide. Their long axes lie parallel to the antero-posterior axis of the squid mantle. Their flat surfaces make angles of between 55 and 60° with the skin surface within which they lie. They consequently lie within a range of angles with the horizontal from approximately 25° at the side of the mantle to 10° near the most ventral parts of the mantle (Fig. 5B).

In white light and when viewed at normal incidence, the ventral iridophores appear red (Fig. 7F). The spectral shifts and polarisation patterns of the ventral iridophores are similar to those described for the mantle 'red' stripe (see Fig. 8). Because of the orientation of the iridophores, however, the light seen when the ventral iridophores are viewed obliquely comes from the inside of the mantle (Fig. 10). In all squid examined, these iridophores were in a reflective state.

The 'blue' stripes

The 'blue' stripes are found in both species of squid between the 'red' stripes and the ventral iridophores (Fig. 5A). The iridophores making up this stripe are orientated with their long axes parallel to the antero-posterior plane of the mantle, and they are between 200 and 400 µm long and 50 µm wide (Fig. 7G). The angle between the horizontal and the normal to the iridophores is approximately 40° (Fig. 5B). The reflections seen at 15° incidence are blue and almost unpolarised (Fig. 7G). At 30° incidence, the reflected wavebands move by approximately 50 nm towards the shorter end of the spectrum
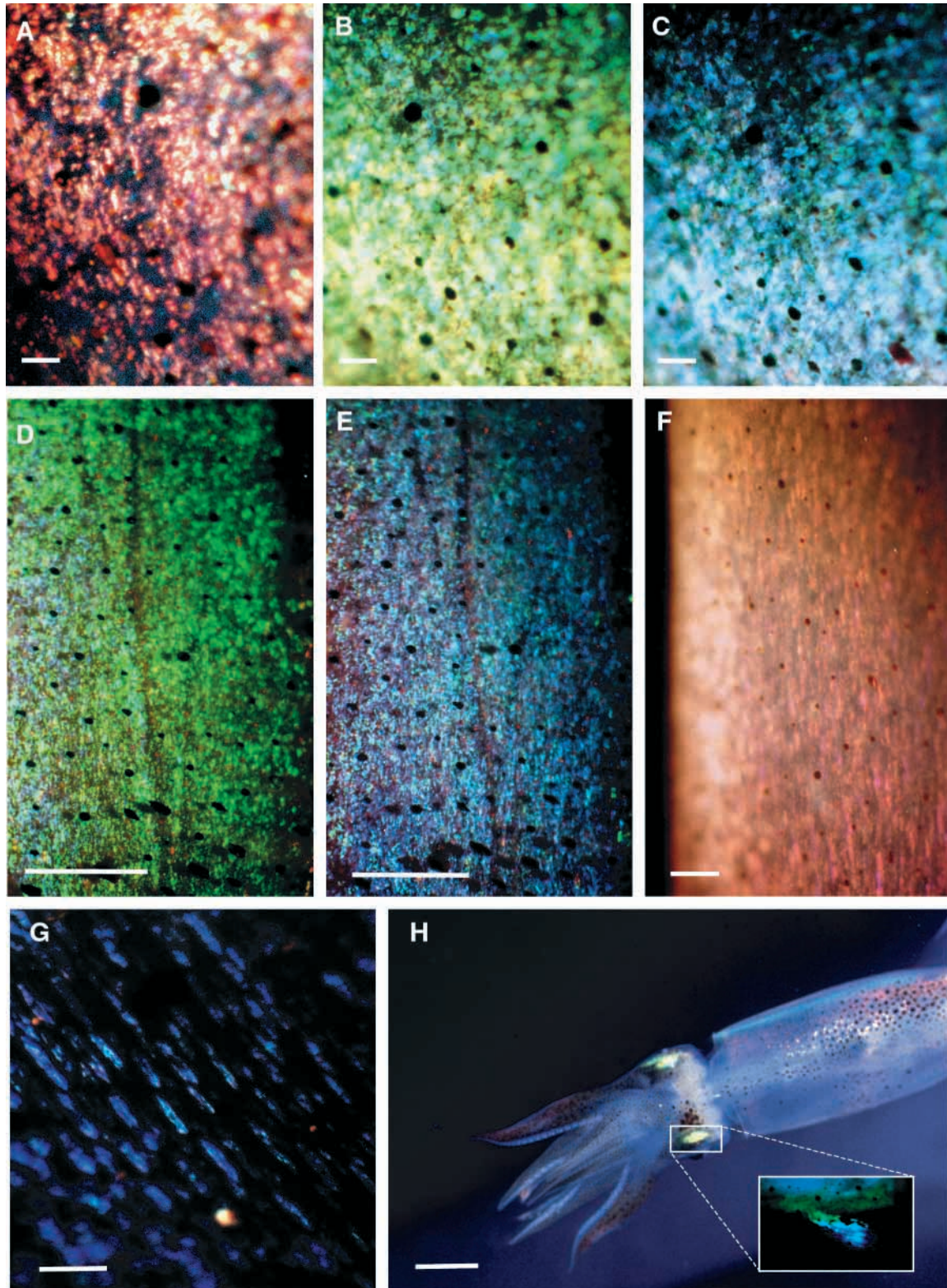
Fig. 7. Photographs of the reflecting stripes of squid. The 'red' stripe at the anterior end of the mantle in white light at (A) 15° incidence, (B) 45° incidence and (C) 55° incidence. Scale bars, 300 μm. The mantle 'green' stripe in white light at (D) 15° incidence and (E) at 45° incidence. Scale bars, 5 mm. (F) The ventral iridophores in white light at 15° incidence. Scale bar, 200 μm. (G) The mantle 'blue' stripe in white light at 15° incidence. Scale bar, 200 μm. (H) Photograph in white light showing the position of the fluorescent layer above the eyes of a squid (*Alloteuthis subulata*). The inset photograph shows the fluorescent layer excited with blue light. Scale bar, 1 cm.

(Fig. 11). The reflections of this stripe disappear at an angle of approximately 35–40° incidence.

Fluorescent and iridescent layers above the eyes

The fluorescent layers

In both species examined, the fluorescent layers can cover all or part of the other reflecting layers of the 'eyespots'. When viewed in white light, these layers emit yellow-green light (Fig. 7H), and the colour of the emitted light does not change with angle of view and it is not polarised. They look equally bright from all angles of view and approximate to being perfect diffusers. In blue light, the fluorescent layers fluoresce in the green parts of the spectrum, at around 500 nm (Fig. 7H, inset). The wavelengths that excite the fluorescence best were found to be between 400 and 420 nm. The measurements using extracted tissue showed no appreciable change in spectral characteristics after extraction. It was found that the emission peak (λ_{max}) was at 485 nm, with maximum excitation at 415 nm (Fig. 12). These measurements revealed a further excitation peak at around 300 nm, but since spectral irradiance in the sea drops off sharply at wavelengths around 350 nm (Le Grand et al., 1954), this excitation peak cannot play an important role in the fluorescence from this tissue in the sea.

Iridescent layers of the 'eyespots'

The reflecting structures of the 'eyespots' lie parallel to the skin surface within which they lie. *A. subulata* has two very bright reflecting layers above the eyes (Fig. 5A). In white light, one layer reflects green when viewed at normal incidence and the other reflects red. The latter layer has not been found in *Loligo vulgaris*, which has only one reflecting layer that reflects green light when viewed at normal incidence. In *L. vulgaris*, the 'red' component lies underneath the green reflecting layer and only becomes apparent if the green reflectors are physically removed (see also Denton and Land, 1971). With increasing angle of incidence, the reflected wavebands become those of shorter wavelength, with spectral shifts and

polarisation patterns similar to those of the 'red' and 'green' stripes described above.

Discussion

Transparency, λ/4 multilayer reflectors and fluorescence

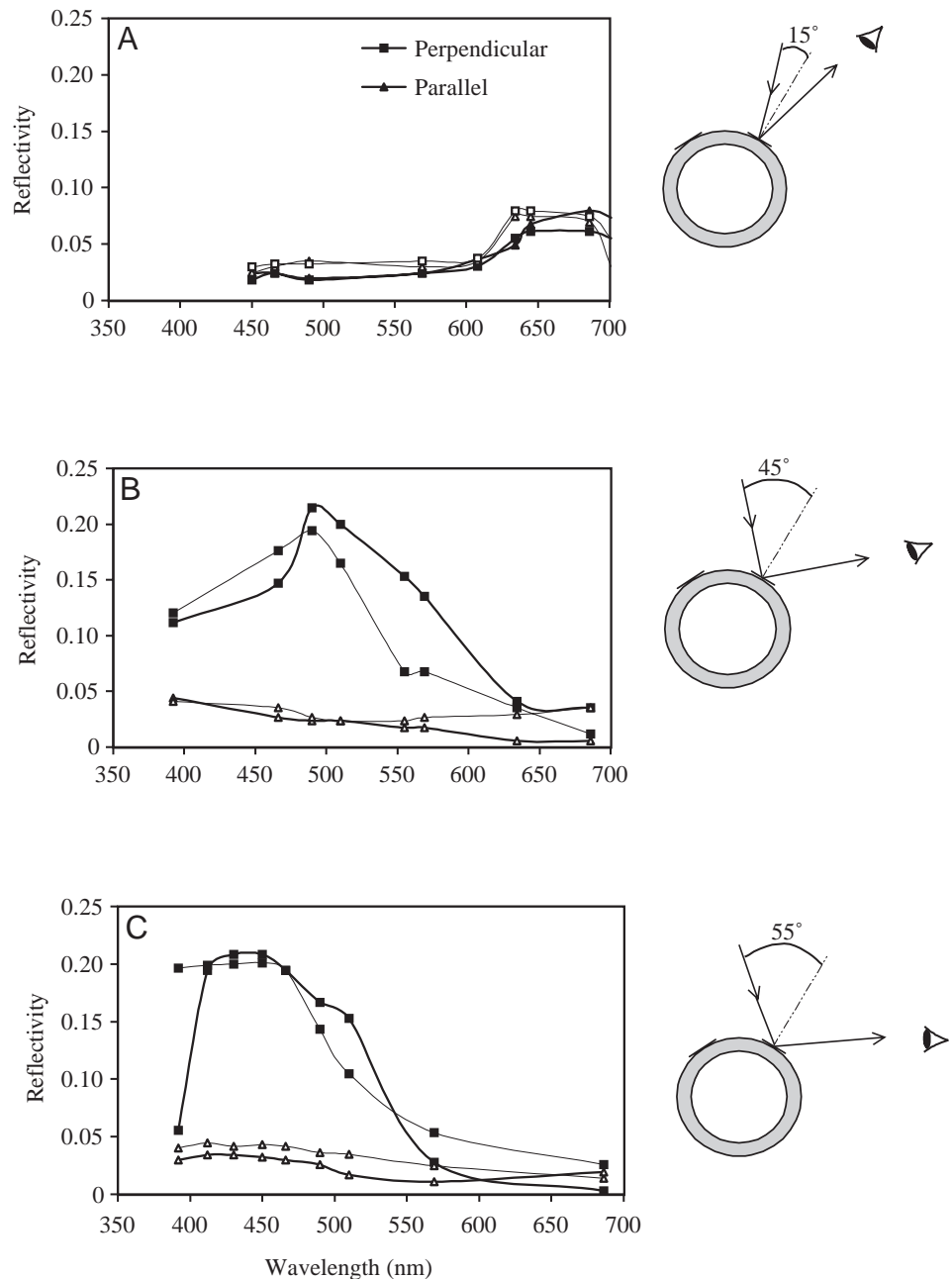When a squid was observed with chromatophores retracted, it could be seen that most parts of the squid are highly

Fig. 8. Spectral reflectivities for the perpendicular and parallel planes of polarisation of the mantle 'red' stripe at a position halfway along the length of the mantle at angles of incidence of (A) 15°; (B) 45° and (C) 55° for *Alloteuthis subulata* (thick lines) and *Loligo vulgaris* (thin lines). The diagrams at the side of each figure show a squid mantle in dorso-ventral section. They indicate the direction in which the light is reflected, i.e. the angle of incidence at which the measurements were made.

transparent. In the midwater environment, largely transparent animals may be quite effectively camouflaged against predation (for a review, see McFall-Ngai, 1990). It may, however, be more difficult for a transparent squid to send visual signals to neighbouring squid without a compromise to the animal's transparency. The reflective stripes of the two species of squid studied have properties that represent a compromise between transparency and signalling. Since the squid disturb the general patterns of light around them very little, even small reflections can give clear signals.

The reflective properties of all the reflective stripes, except those of the fluorescent layers, resemble those of λ/4 stacks. The bandwidth of the reflections, their spectral shifts with changes in angle of incidence and the polarisation patterns measured for the various iridophore stripes are in good agreement with the theoretical predictions of Huxley (Huxley, 1968) and Land (Land, 1972) (see Fig. 4). This agrees with the findings of Denton and Land (Denton and Land, 1971), who showed that the chitin plates from the reflectors above the eyes of *L. forbesi* have optical thicknesses approximating to a quarter of the wavelength of the light that is best reflected at normal incidence. It has been shown by transmission electron microscopy that the iridophore platelets of some squid are arranged in stacks (Arnold, 1967; Mirow, 1972; Arnold et al., 1974) and that they have optical thicknesses approximating those expected for λ/4 reflectors (Cooper et al., 1990). Hanlon et al. (Hanlon et al., 1983) performed experiments in which they shone a beam of white light onto the anterior end of the 'red' stripe and found that, when viewed from various angles, the preparation became coloured. They argued that thin-film devices do not produce a spectrum of colours with a given angle of incident light and that the edges of the iridophores act as diffraction gratings (see also Cloney and Brocco, 1983). We found that the changes in colour with changes in angle of incidence and the polarisation properties agree well with the theory of λ/4 stacks. From the measurements using aluminium foil, it is expected that the iridophores

of the 'blue' stripes and the ventral side are orientated with their flat surfaces at an angle to the skin surface so that the platelets lie edge on to the skin surface. The results we report here suggest that the colours are produced by constructive interference of light reflected from the platelets rather than by diffraction from the edges of the platelets.

On the 'eyespots', we found two types of reflector, one with the properties of λ/4 stacks, the other fluorescent and with the properties of a diffuser. The iridophore stripes underneath the fluorescent layer will act as a tapetum, reflecting green and blue
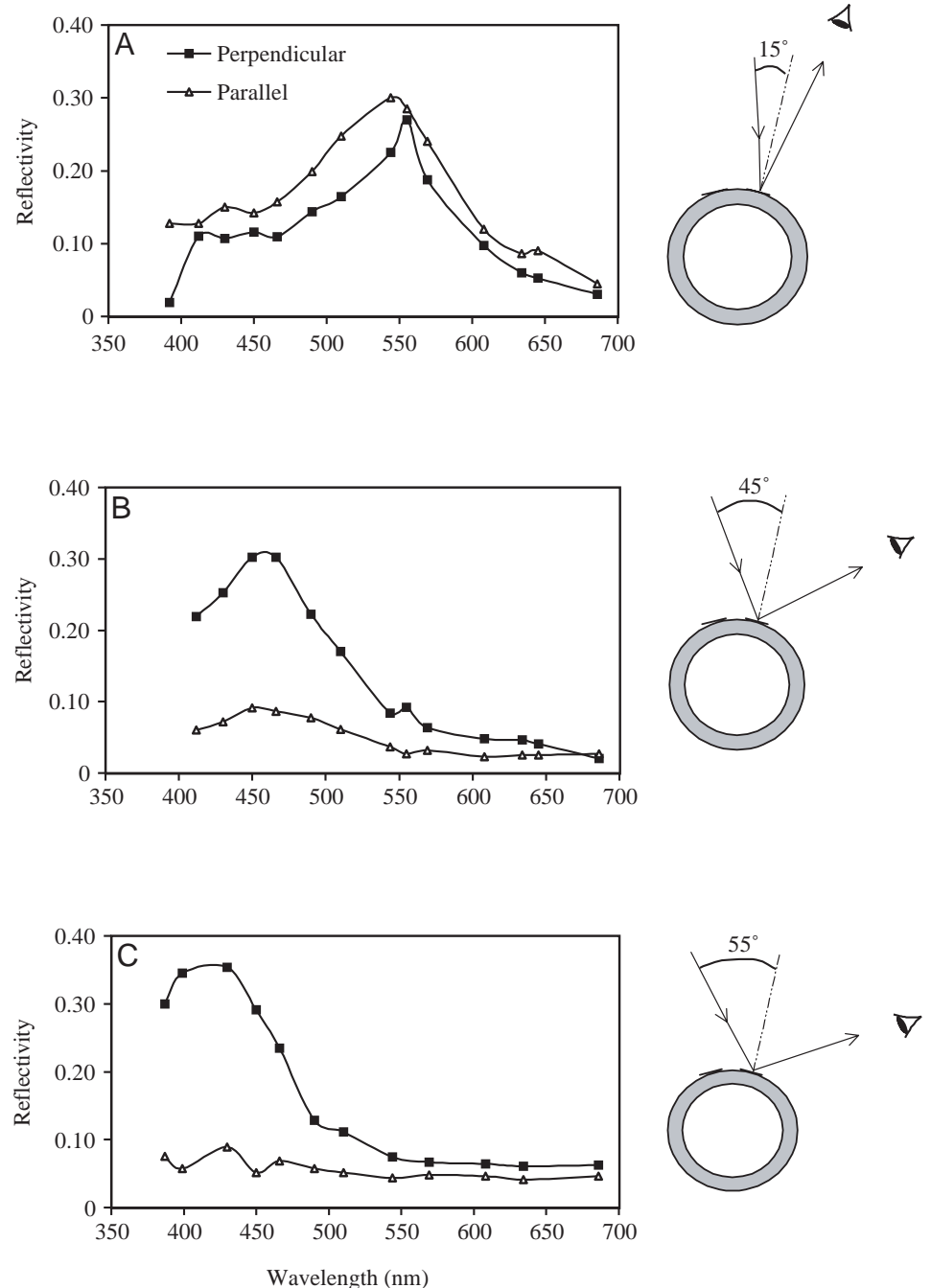

Fig. 9. Same as Fig. 8 but for the mantle 'green' stripe of *Loligo vulgaris* at (A) 15°, (B) 45° and (C) 55° incidence.
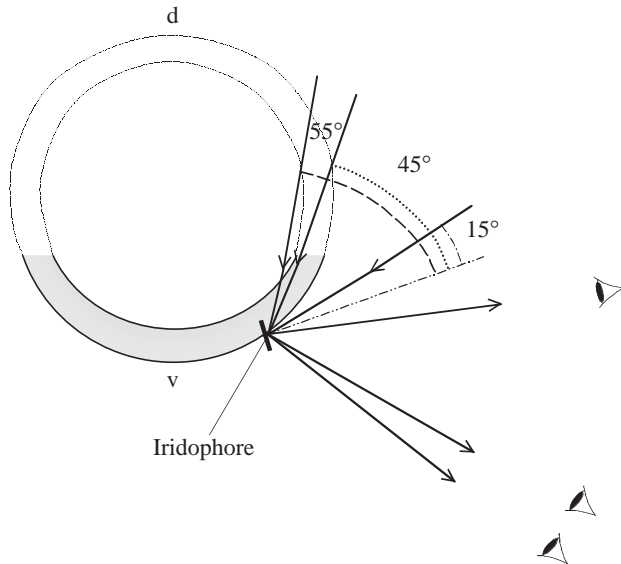
Fig. 10. Squid mantle in dorso-ventral section (d and v represent dorsal and ventral regions) showing the directions in which light is reflected from the iridophores of the ventral side. Dashed double-dotted line, normal incidence to iridophore; solid line, incident and reflected light; dashed dotted line, 15° incidence; dotted line, 45° incidence; dashed line, 55° incidence.

light, whose energy is transferred to the fluorescent layer and emitted as green light. The result of the fluorescence is that a certain number of quanta corresponding with light in a waveband around 415 nm are transformed to light in a waveband around 485 nm. Although the quanta produced are smaller than those exciting the fluorescent layers, they will be approximately 10 times more effective at bleaching the photosensitive pigment of the squid.

Iridophore reflections in the light environment of the sea

Although some squid iridophores reflect light in the red part of the spectrum, the intensities of these reflections in the sea will be relatively small because of the low intensity of the red light available (see Fig. 1A). It is thought that the squid used in our study have only one visual pigment absorbing maximally at around 500 nm (Morris et al., 1993). A pigment of this kind will have very low absorption in the red, and the eyes of the squid will be very insensitive to such light. Although, at near-normal incidence, the red stripe iridophores reflect red light, at higher angles of incidence the reflections become those of shorter (green and blue) wavelengths. These are the wavebands that penetrate best into the sea and to which the eyes of these squid are most probably sensitive. Fig. 13 shows how large these effects can be. Fig. 13A summarises the results on spectral reflectivity (R_λ) obtained for the 'red'

stripe. Fig. 13B shows the effects of the spectral intensity of daylight in the sea (E_λ) (in the example given here at a depth of 19 m) and the spectral sensitivity of the photosensitive pigment of A. subulata on the reflections seen by these squid (see Ratio 1 below). It may be seen from this figure that, even at depths of a few metres, the reflections from the iridophores will be confined largely to the blue-green parts of the spectrum, and these will be much more effective than other wavebands in stimulating the squid's visual pigment. The 'blue' and 'green' stripes reflect best in the blue-green at low angles of incidence, so that the reflections are most effective at these angles. The fluorescent layers emit blue-green light at both normal and oblique angles of incidence.

The spectral irradiance and visual pigment curves shown in Fig. 1A are almost identical and, as a consequence, the effective reflections perceived by the receptors containing this pigment will not change appreciably when the squid swim in shallow water in daylight. It remains possible that squid can perceive colour. Although having one photosensitive visual pigment suggests that an animal is colour-blind, we may recall that, by using stable screening pigments or by using the visual pigment itself as a screen, colour vision is possible (see, for example, Denton and Locket, 1989). At shallow depths, at which the intensities of light of longer wavelengths are high, colour vision would be advantageous, since the reflections are highly coloured.

At angles around Brewster's angle, the reflections from the iridophore stripes are highly polarised. Both the plane and the degree of polarisation change dramatically depending on the movement of the squid. For schooling squid, this could be a powerful information source, because cephalopods have the ability to discriminate light polarised in different planes (Moody and Parriss, 1960; Saidel et al., 1983; Shashar et al., 1996; Shashar et al., 2000). The polarisation patterns are complicated. Near the surface of the sea, they are influenced by the changing patterns of polarisation in the sky. These are mainly ascribed to scattering of directional light, as a result of which the orientation of the e-vector will depend on the direction of view relative to the bearing of the sun, the sun's altitude and the depth of the water
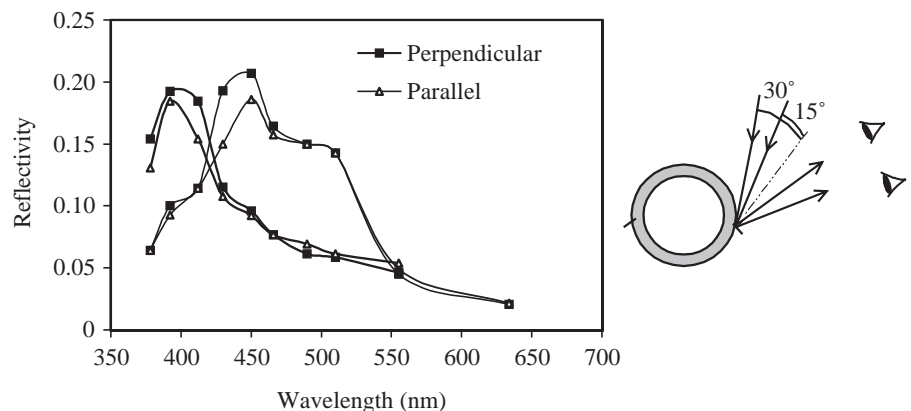

Fig. 11. Same as Fig. 8 and Fig. 9 but for the mantle 'blue' stripe of Loligo vulgaris at (thick line) 15° and (thin line) 30° incidence.
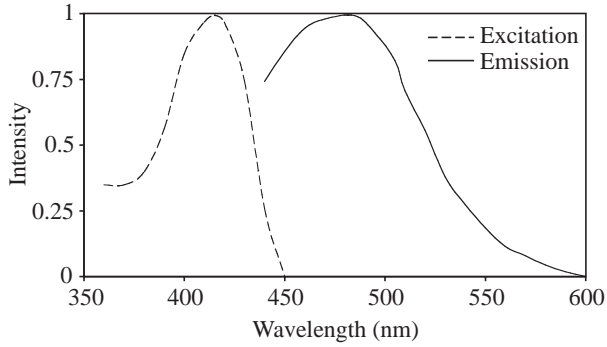
Fig. 12. The excitation and emission spectra of an ethanol pigment extract of the fluorescent layers of *Loligo vulgaris*. Intensity has been normalized to the maximum value for each spectrum.
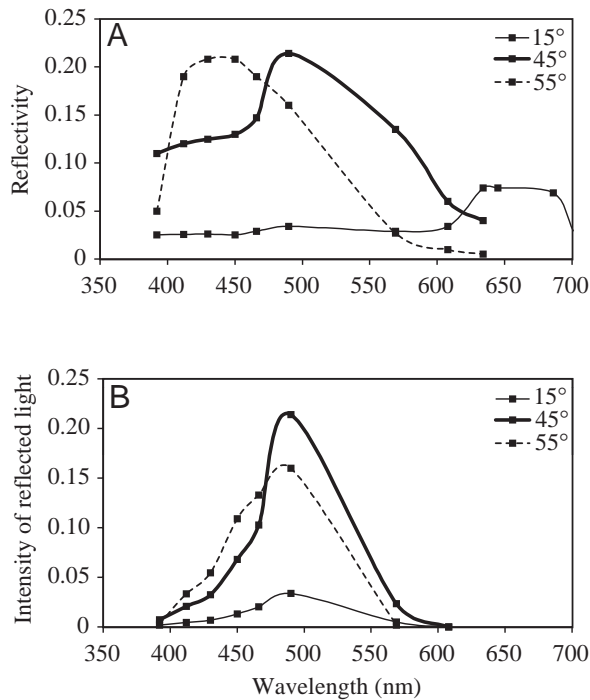

Fig. 13. (A) Reflectivity (R_λ) of the mantle 'red' stripe in the perpendicular plane of polarisation (see Fig. 8). (B) Ordinate is $E_\lambda R_\lambda S_\lambda$, where E_λ is the spectral irradiance of submarine daylight, R_λ is the reflectivity shown in A and S_λ is the spectral absorption of the squid's rhodopsin (see Fig. 1A for E_λ and S_λ).

(Waterman, 1954; Waterman, 1955; Waterman and Westell, 1956). Generally, the degree of polarisation is between 5 and 35 % (Waterman and Westell, 1956; Ivanoff and Waterman, 1958; Ivanoff, 1956). In one exceptional case, Ivanoff (Ivanoff, 1959) found values of polarisation as high as 85 %.

Visibility and invisibility in the sea

In Fig. 14 we show, as an example, a squid mantle in cross section and an observer looking at a reflective area on the surface of the squid. The visibility of the reflecting area to the observer will depend on a number of factors: (i) the radiance
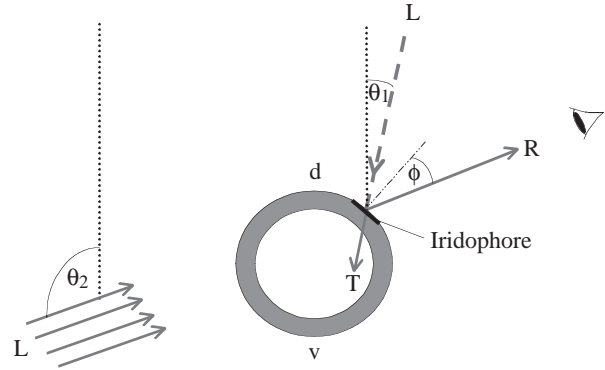

Fig. 14. Diagram to illustrate the meaning of the symbols used in Ratio 1, showing a squid mantle in dorso-ventral section (d and v represent dorsal and ventral regions) and an observer looking at a reflective area on the surface of the squid. Dotted line, vertical line; dashed line, light (L) incident to the iridophore at an angle θ_1 with the vertical; dashed double-dotted line, normal incidence to the iridophore; solid line, reflected (R) and transmitted (T) light at an angle of incidence ϕ to the normal of the iridophore; the group of four arrows indicates the background light field at an angle θ_2 with the vertical.

of the light from the direction from which the reflections arise ($L_{\theta 1}$), (ii) the radiance of the background light field against which the area is viewed ($L_{\theta 2}$), (iii) the spectral reflectivity of the reflector ($R_{\lambda\phi}$) at an angle of incidence ϕ, (iv) the spectral irradiance of submarine daylight (E_λ) and (v) the spectral sensitivity of the eye of the observer (S_λ). The intensities of the reflections with respect to the backgrounds against which they are viewed are given by the ratio

$$L_{\theta 1}\int_{400\,\mathrm{nm}}^{700\,\mathrm{nm}}(E_\lambda R_{\lambda\phi} S_\lambda \delta\lambda) : L_{\theta 2}\int_{400\,\mathrm{nm}}^{700\,\mathrm{nm}}(E_\lambda S_\lambda \delta\lambda). \qquad (1)$$

We assume that, at a given depth, the spectral distribution of energy in the sea is the same in all directions of view (see upwelling and downwelling irradiance measurements of Tyler and Smith, 1970). We use (i) the values for the angular distribution of light given by Denton et al. (Denton et al., 1972), after the equation of Tyler (Tyler, 1963) (Fig. 1B) and (ii) the spectral irradiance data from Tyler and Smith's (Tyler and Smith, 1970) measurements in the Gulf of California (19 m depth) (Fig. 1A). (iii) The absorption curve S_λ of the squid's visual pigment (taken as the measure of spectral sensitivity) was calculated after Knowles and Dartnall (Knowles and Dartnall, 1977) with $\lambda_{\max}$=499 nm, which is the wavelength at which the rhodopsin of *A. subulata* absorbs best (Morris et al., 1993) (Fig. 1A).

The mantle 'red' stripe

Fig. 15A shows diagrammatically how the orientations and the reflective properties of the 'red' stripe iridophores will affect the visibility of the 'red' stripe to three observers (X, Y and Z) at different positions in a plane perpendicular to the long axis of the squid. It may be seen from this figure that a 'red' stripe
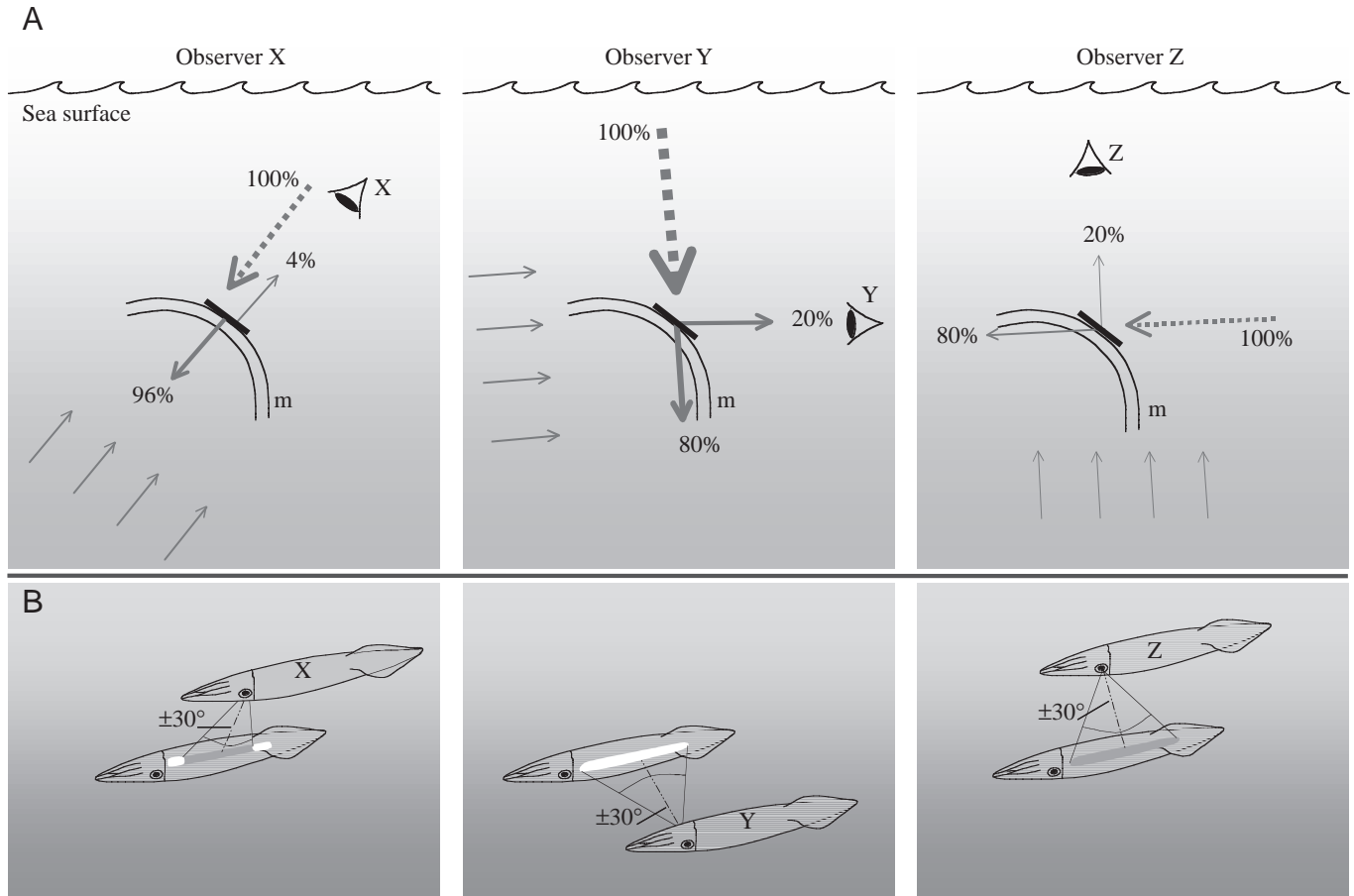
Fig. 15. (A) Iridophore of the mantle 'red' stripe (see Fig. 5B) showing three observers (X, Y and Z), each viewing reflections of light from the iridophore. The line styles of this figure and the symbols given in the text are the same as shown in Fig. 14. The thickness of each line roughly indicates intensity, i.e. the thicker the line, the greater the intensity. While the reflections seen by observer X are unpolarised, those seen by observers Y and Z are maximally polarised. m, mantle. (B) The reflective patterns seen looking along the length of the mantle 'red' stripe. The shading of the 'red' stripe indicates intensity of reflection, i.e. the lighter the shading, the higher the intensity. Dotted lines indicate the position of the eye of the observer opposite the midpoint of the 'red' stripe. Observer X: within an angle of view of less than ±30°, the intensities of the reflections will equal the background light. For a larger angle of view, the reflections (shown in white) become brighter than the background and polarised. Observer Y: the reflections seen within an angle of view of approximately ±30° will be polarised and approximately four times brighter than the background. Observer Z: the brightness of the reflections will equal the background light over the entire visual field.

iridophore reflects light that always arises from a direction in which the radiance is much higher than that of the background. Observer X, for example, located in a plane normal to the surface of the iridophore ($\phi=0°$), will compare the unpolarised reflections arising from an angle of $\theta_1=40°$ to the downward vertical with the approximately 30 times dimmer background at an angle of $\theta_2=140°$. The fraction of the light reflected at near-normal incidence ($15°$ incidence) in the blue-green, calculated from the experimental results shown in Fig. 8A, is approximately 4%. Assuming that the angular distribution of light in the sea is of the type shown in Fig. 1B, we find that these reflections are sufficient to approximately equal the background light. Observer Y (Fig. 15A) in a position $\phi=45°$ to the normal of the iridophore, will compare the heavily polarised reflections arising from an angle of $\theta_1=5°$ to the vertical with the 40 times dimmer background light at an angle of $\theta_2=95°$ with the vertical. At $45°$ incidence, approximately 20% of the light striking the

iridophore in the perpendicular plane of polarisation is reflected. Assuming that the background light is largely unpolarised and that the squid sums both planes of polarisation, we calculate that the reflections are approximately four times brighter than the background. If the squid eye treats the planes of polarisation separately, the reflections in the perpendicular plane of polarisation will have an intensity eight times that of the background, whilst those in the parallel plane of polarisation have an intensity lower than that of the background. Observer Z (Fig. 15A) will compare the heavily polarised reflections arising from an angle of $\theta_1=85°$ to the vertical with the background from an angle of $\theta_2=175°$. This observer is also positioned at $\phi=45°$ to the normal of the iridophore, and we find that these reflections are enough to approximately equal the background light.

So far, we have only considered reflections in the plane perpendicular to the long axis of the squid. However, the

reflective patterns depend greatly on the distances and directions from which the observers view the stripe. In Fig. 15B, we show for observer X, opposite the midpoint of the 'red' stripe at a distance of half a squid's mantle length, changes in visibility along the length of the 'red' stripe. For an angle of view between the observer and the surface of the squid of less than ±30°, reflectivity from the iridophores in the blue-green is low and the reflections are weakly polarised (red light is strongly reflected but, at the depth assumed here, the intensity of red light is low). For a larger angle of view, we find that the reflections in the blue-green become brighter than the background and polarised. We may note that the angle of incidence to the iridophores is greatly affected by the anatomy of the lozenge-shaped units of the 'red' stripe. To give an example, if these units lay parallel to the skin surface, an angle of view of ±30° would mean that the angle of incidence to the iridophores seen at the periphery would be 30°. At this angle of incidence, reflectivity in the blue-green would be low and the reflections would be unpolarised. The two pairs are, however, tilted with respect to the skin surface, so that the angle of incidence to one pair is increased by 10–15°, which makes it 40–45°, rather than 30°, while the angle of incidence to the other pair is decreased by 10–15°, which makes it 15–20°. For the former pair, reflectivity will therefore be higher than for the latter pair, so that the reflections of half of the unit seen towards head and 'tail' will be much brighter and more polarised than the units of the mid-mantle.

On the basis of trigonometric relationships, we can calculate that, for observer Y in Fig. 15B, the reflections of the polarised light seen within an angle of view of ±30° will appear at least four times brighter than the background. Both reflectivity and degree of polarisation will decrease with an angle of view greater than that, since the angle of incidence to the iridophores becomes larger than 60°, at which the reflections will be maximal in the near ultraviolet. The intensity of ultraviolet relative to blue-green light diminishes with increasing depth (Le Grand et al., 1954), so that the reflections in this part of the spectrum become limited by the weakness of the ultraviolet light present. The same trigonometric calculations show that the reflections seen by observer Z (Fig. 15B) will approximately match the background radiance over the entire visual field, thus making the squid hard to detect.

The mantle 'blue' and 'green' stripes and the ventral iridophores

The iridophores of the mantle 'blue' stripe can be seen only when viewed from the side and, for the examples given above, only observers X and Y will see this stripe. This is because these iridophores only reflect light visible to us for a small range of angles around normal incidence. At high angles of incidence, we expect that the reflections will be strongest in the ultraviolet. Again, the iridophores of the 'blue' stripe reflect light arising from a direction in which the light is much brighter than the background and, despite their low reflectivity in the blue-green (15%), the stripes will appear approximately four times brighter than the background.

The 'green' stripes found between the fins of *L. vulgaris* and the 'green' patches found anterior to the 'green' stripes give very bright blue-green reflections both upwards and sideways. These reflections may be useful signals to other squid. We have observed on a number of occasions how *L. vulgaris* males show the 'accentuated testis' component (described by, for example, Hanlon et al., 1999), in which the 'green' stripes appear as conspicuous flashes in an area in which the chromatophores are retracted.

Midwater animals are most visible when seen from below against the background of the strongest radiance in the sea. If the light travelling downwards is absorbed by the animal, the loss cannot be replaced by reflections of light from any other direction. Being transparent makes a midwater animal less likely to be detected by predators and in comparison with other muscular animals, such as herring and mackerel, the squid that were the subjects of the present study are very transparent. The loose spacing of the 'red' stripe iridophores, their low reflectivity and high blue-green transmission allow approximately 90% of the incident light to be transmitted through the skin. Despite opacity changes that occur during mantle muscle activity (Abbott and Lowy, 1956), we found that the mantle muscle itself transmits approximately 95% of blue-green light and that at most 30% of the transmitted blue-green light is lost in the internal organs. The iridophores of the ventral side are orientated so that the blue-green light that enters the mantle cavity falls on them obliquely (Fig. 16) and
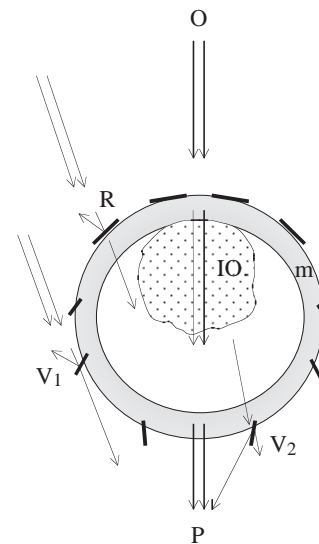

Fig. 16. Cross section through the mantle of a squid (from Fig. 5B) showing how squid may maximise camouflage to observers from below. A large fraction of the incident light available at point O will pass through the spaces between the iridophores, through the mantle muscle (m) and through the internal organs (IO) to point P. Only a small fraction of the light is reflected by the iridophores (e.g. the 'red' stripe, at point R), allowing a large fraction to be transmitted into the mantle cavity. The ventral iridophores (V_1) reflect weakly at angles around normal incidence, allowing a large part of the light to be transmitted. At oblique angles of incidence (V_2), the ventral iridophores have high reflectivity and low transmission, channelling the incident light downwards.
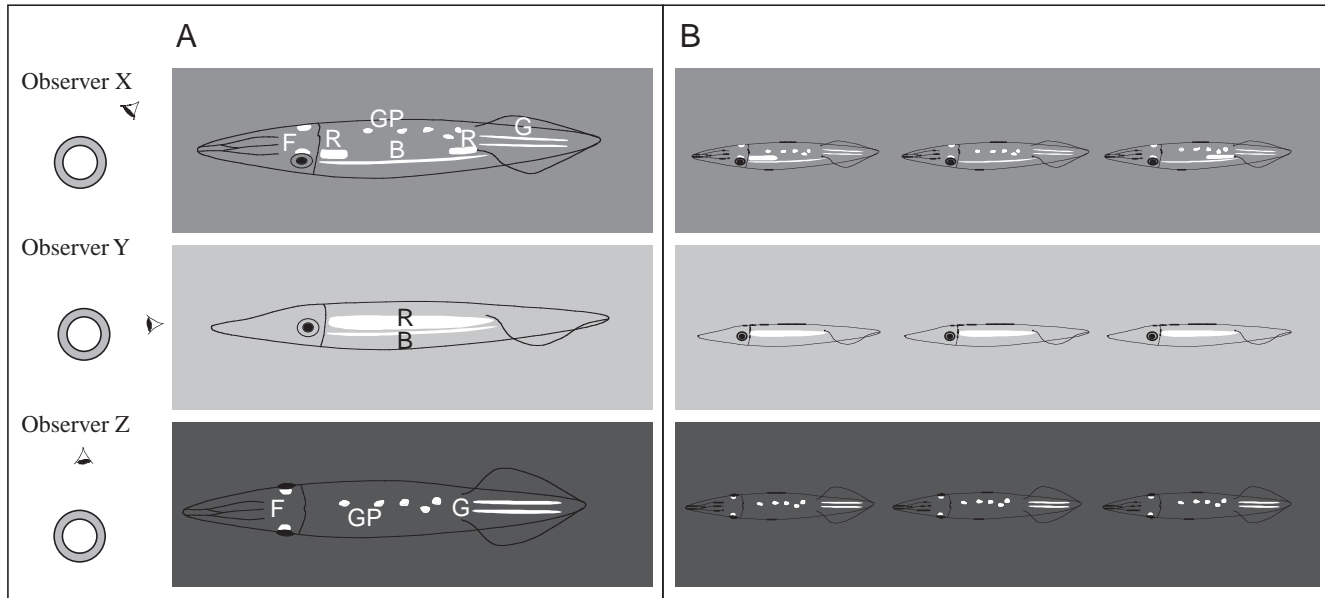
Fig. 17. The effects of position on the reflective patterns seen by observers X, Y and Z (see Fig. 15). (A) At a distance of half a squid's mantle length. F, fluorescent layers; R, 'red' stripe; B, 'blue' stripe; G, 'green' stripes; GP, 'green' patches (G and GP only for *L. vulgaris*). (B) For observers at a distance of 1.5 mantle lengths.

so the light is channelled downwards by the ventral iridophores, which reflect light strongly at oblique angles of incidence. At normal incidence, at which reflectivity in the blue-green is low, the iridophores transmit the incident blue-green light very efficiently, so that the total light directed downwards minimises the shadow cast to an observer below the animal.

The fluorescent layers and reflectors above the eyes

The fluorescent layer and the underlying iridophores have relatively high reflectivities, so it appears very likely that they play some role in visual communication. Since squid steer by turning their head and arms, it seems likely that the reflections from both 'eyespots' may give an early indication of changes in movement of schooling squids. In some respects, the 'eyespots' are similar to the reflectors on both sides of the tail of many fish. The reflections of light by these 'tailspots' can give strong visual stimuli to neighbours with certain movements of the fish (Denton and Rowe, 1994). The 'eyespots' are very bright, so the squid are in danger of being detected by predators from above. By expanding overlying chromatophores, the squid can eliminate this hazard.

The effects of position on the visibility of the reflective stripes

In Fig. 17A we illustrate the reflective patterns of all the iridophore stripes observed from the positions of observers X, Y and Z. Observer X will see the fluorescent 'eyespots', the bright and polarised reflections at the anterior and posterior ends of the 'red' stripe, the unpolarised reflections from the 'blue' stripe and the polarised reflections from the 'green' stripes and 'green' patches. While observer Y will only see the bright and polarised reflections of the 'red' stripe and the

unpolarised reflections from the 'blue' stripe, observer Z will see the fluorescent 'eyespots' and the unpolarised reflections from the 'green' stripes and 'green' patches.

No observations have so far been made on the relative distances between schooling *L. vulgaris* and *A. subulata* in the field. A study on a related squid, *Sepioteuthis lessoniana*, showed that in captivity these squid school at 1.6 body lengths (Boal and Gonzalez, 1998). The reflective patterns described above were calculated for squid schooling at distances of half a mantle length, a distance that has been recorded for a school of *A. subulata* in the Plymouth laboratory (Dr P. Lima, unpublished data). Evidently, the reflective patterns will change with increasing distance between the observer and the squid (Fig. 17B). If, for example, observer X views the squid from a distance of 1.5 mantle lengths, the two bright spots at the anterior and posterior ends of the 'red' stripe might indicate the presence of two additional squid, one ahead and one behind the observer. Observer Y at a distance of 1.5 mantle lengths will only see the 'red' stripe, since the angle of incidence to the blue stripe is too high, and for observer Z at this distance the reflective patterns over the length of the animal remain unchanged.

Changes in the orientation of a squid with respect to the light field around it will change the brightness, position and polarisation of the reflective stripes. If, for example, the squid rolls, one of the 'red' stripes will become brighter than the other; if the squid yaws, the stripes will appear longer, or shorter, depending on the position of the observer.

It would be advantageous for the squid to be able to detect the differences in brightness of individual iridophores. The iridophores are generally approximately 200 μm in length. In a squid with a length of 30 cm, the visual acuity needed to resolve iridophores of 200 μm at a distance of 15 cm would

be 0.2 (i.e. approximately 5′). This is 10 times less than the highest acuity for man (Pirenne, 1948). Even allowing for the fact that the eye of *L. vulgaris* is approximately half the size of the human eye, we might expect that its acuity would be sufficient to enable the squid to resolve these iridophores.

Activity of iridophores and the effect of chromatophore activity

Some of the iridophore stripes found in *L. vulgaris* and *A. subulata* are not in a reflective state at all times. They vary from being non-reflective to weakly or strongly reflective. This has also been described by Hanlon (Hanlon, 1982), who observed that some iridophores of the squid *Loligo plei* are not visible at all times. It has been reported that the spectral reflectivities of some iridophores of the squid *Lolliguncula brevis* change in response to topical application of acetylcholine, with reflections becoming those of shorter wavelengths with increasing concentrations of acetylcholine (Cooper and Hanlon, 1986; Cooper et al., 1990; Hanlon et al., 1990). This wavelength shift has not been observed in *L. vulgaris* and *A. subulata*. It has, however, been possible to 'retrieve' iridophore reflections, several hours after they had disappeared, by perfusing specimens with a solution of $50\,\mu\mathrm{mol}\,l^{-1}$ carbachol (Sigma). One of us (L.M.M.) has observed that under stress (e.g. during capture of a squid) the mantle 'red' stripes become very prominent, suggesting that iridophores could be controlled by the animal. The effect that iridophore activity would have on the reflectivities described in this paper would be to increase or decrease the intensity of the reflections with respect to the background radiance.

The iridophore stripes are overlain by hundreds of chromatophores, which can be observed in a variety of retraction states, producing a wide range of body patterns. The interaction between chromatophores and iridophores and their importance for camouflage and visual signalling is outside the scope of the present work and will be the subject for future study. It appears likely, however, that chromatophores can cover the reflections from the iridophore stripes. Their pigments are commonly of longer wavelengths (red, yellow and brown) and will absorb light in the blue-green parts of the spectrum. This will have the effect of making the squid darker if the chromatophores expand, reducing iridophore reflectivity; conversely, their retraction will increase reflectivity from the iridophore stripes.

Concluding remarks

In this paper, we report the existence of distinct iridophore stripes in squid. The stripes are described in terms of their spectral reflectivity, polarisation and orientation. Our measurements show that the transparency of the squid mantle and the ventral iridophores may minimise the visibility of a squid to observers below the animal. The iridophores of the ventral side have high reflectivity in the blue-green at oblique angles of incidence. This will channel the light, which passes through the mantle muscle, downwards, so that the squid minimises the shadow cast below the animal.

The fluorescent layers and the iridophores of the 'red', 'green' and 'blue' stripes are orientated in such a way that they reflect light, which always arises from directions in which the radiances are higher than those of the background. They consequently disrupt, rather than aid, camouflage and it seems that their function lies in communication between members of the same species, e.g. signalling between neighbours in schools.

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